Molecules and Cells

# CTRP9 Regulates Growth, Differentiation, and Apoptosis in Human Keratinocytes through TGFβ1-p38-Dependent Pathway

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http://dx.doi.org/10.14348/molcells.2017.0097
www.molcells.org

Impairment of wound healing is a common problem in individuals with diabetes. Adiponectin, an adipocyte-derived cytokine, has many beneficial effects on metabolic disorders such as diabetes, obesity, hypertension, and dyslipidemia. C1q/TNF-Related Protein 9 (CTRP9), the closest paralog of adiponectin, has been reported to have beneficial effects on wound healing. In the current study, we demonstrate that CTRP9 regulates growth, differentiation, and apoptosis of HaCaT human keratinocytes. We found that CTRP9 augmented expression of transforming growth factor beta 1 (TGFβ1) by transcription factor activator protein 1 (AP-1) binding activity and phosphorylation of p38 in a dose-dependent manner. Furthermore, siRNA-mediated suppression of TGFβ1 reversed the increase in p38 phosphorylation induced by CTRP9. siRNA-mediated suppression of TGFβ1 or p38 significantly abrogated the effects of CTRP9 on cell proliferation and differentiation while inducing apoptosis, implying that CTRP9 stimulates wound recovery through a TGFβ1-dependent pathway in keratinocytes. Furthermore, intravenous injection of CTRP9 via tail vein suppressed mRNA expression of Ki67 and involucrin whereas it augmented TGFβ1 mRNA expression and caspase 3 activity in skin of type 1 diabetes animal models. In conclusion, our results suggest that CTRP9 has suppressive effects on hyperkeratosis, providing a potentially effective therapeutic strategy for diabetic wounds.

Keywords: apoptosis, C1q/TNF-Related Protein 9, differentiation, p38, proliferation, transforming growth factor beta 1

## INTRODUCTION

Adiponectin is a well-known adipokine that has been recognized as a key regulator of insulin signaling and inflammation (Jee et al., 2013; Whitehead et al., 2006). Recently, adiponectin has been reported to ameliorate the impaired wound healing associated with diabetes (Kawai et al., 2008). C1q/TNF-Related Protein (CTRP9) is the closest paralog of adiponectin. Its biochemical functions, including specific expression in adipocytes, endocrine function, and formation of higher-order oligomeric complexes, are similar to those of adiponectin due to their structural similarity (Peterson et al., 2013). CTRP9 is also known to be an adipokine involved in communication among skeletal muscle, liver, and adipose tissue (Peterson et al., 2013). CTRP9 transgenic mice showed improvements in fasting blood glucose and insulin levels, weight loss, and fatty liver (Peterson et al., 2013). Conversely, CTRP9 knockout mice showed weight gain, insulin resistance, and hepatic steatosis (Wei et al., 2014). In a clinical study, serum CTRP9 level was inversely correlated with age, blood pressure, and the lipid profile associated

Received 15 June, 2017; revised 1 November, 2017; accepted 5 November, 2017; published online 16 November, 2017

eISSN: 0219-1032
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with insulin resistance and positively correlated with serum total adiponectin level (Hwang et al., 2014). Although several studies have reported the influence of CTRP9 on metabolic syndrome, its effects on impaired wound healing, which is commonly seen in diabetic foot, remain unclear.

Impaired wound healing is observed in patients with diabetes and results in problems such as hyperkeratinization and callosity around diabetic ulcers. Keratinocytes are important cellular components of the epidermis and play a crucial role in the wound healing process through re-epithelialization. Elevated cell proliferation and differentiation are commonly observed in patients with diabetic foot (Usui et al., 2008). Apoptosis in keratinocytes plays a crucial role in the regulation of epidermal development through morphological and biochemical changes. Impaired wound healing in diabetes is caused by diminished keratinocyte apoptosis (Blakytny and Jude, 2006). When injured, keratinocytes induce various proteins including growth factors and pro-inflammatory cytokines, which activate fibroblasts and formation of granulation tissue (Bandyopadhyay et al., 2006; Maas-Szabowski et al., 2003; Wang et al., 2006). Therefore, regulation of these molecules for appropriate proliferation, differentiation, and apoptosis in keratinocytes could be an effective therapeutic approach for treatment of impaired wound healing, including diabetic foot ulcer.

In this study, we examined the effect of CTRP9 on human keratinocytes and abdominal skin tissue of experimental mice. Our results demonstrate that CTRP9 suppresses both proliferation and differentiation and induces apoptosis of keratinocytes. CTRP9 also induces expression of transforming growth factor beta 1 (TGFβ1) and phosphorylation of p38 in keratinocytes. These data suggest that CTRP9 plays a crucial role in ameliorating the impaired wound healing in diabetes through a TGFβ1/p38-dependent pathway.

## MATERIALS AND METHODS

### Cell cultures, reagents, and antibodies

Human keratinocyte HaCaT cells (ATCC, USA) were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere of 5% $CO_2$ at 37℃. Mycoplasma was not detected in HaCaT cells. We used HaCaT cells at passages 5-10 for experiments. Human recombinant CTRP9 made by *E. coli* was purchased from Aviscera Bioscience (USA) (Jung et al., 2015; Kambara et al., 2012). Calcium chloride and sodium arsenite were purchased from Sigma (USA). The following antibodies were used in this study: anti-phospho AMPK (1:1000), anti-AMPK (1:3000), anti-phospho p38 (1:1000), anti-p38 (1:2500), anti-phospho JNK (1:1000), anti-JNK (1:1000), anti-phospho ERK1/2 (1:1000), and anti-ERK1/2 (1:3000) were purchased from Cell Signaling (USA); anti-Ki67 (1:100) and anti-involucrin (1:100) were obtained from Abcam (USA); anti-beta actin (1:5000) was obtained from Santa Cruz Biotechnology (USA).

### Cell proliferation assay

BrdU kit (Roche, Germany) was used according to manufacturer's direction. In brief, cells were seeded into 96-well plates at a density of $2.5 \times 10^4$ cells/well and incubated overnight, followed by serum starvation for 12 h. Next, the cells were treated with various concentrations of CTRP9 for an additional 24 h, then pulse labeled with 5-bromo-20-deoxyuridine (BrdU) for 2 h. Finally, absorbance at 370 nm was measured using a multi-reader. Proliferative cells were visualized using a mouse monoclonal anti-human Ki67/FITC antibody (Thermo Fisher Scientific, USA).

### Animals, feeding, treatment, and wounding

This study was approved by the institutional animal review board (Institutional Animal Care and Use Committee of Bundang Seoul National University Hospital, Korea). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication, 8th edition, 2011). A control group (n = 5) and two experimental groups (5 animal each) of 8-week-old male C57BL/6J (B6) mice were treated with a normal diet (ND; Brogaarden, Denmark). Hyperglycemia was induced by a single intravenous injection of 150 mg/kg streptozotocin (STZ) (Sigma) to establish a type 1 diabetic animal model. Mouse recombinant CTRP9 was injected to mice via tail vein (0.5 μg/g mice/2 days) for 2 weeks. To evaluate the wound healing response, all experimental mice were subjected to a dermal punch biopsy. After wound healing, mice were anesthetized, the fur was shaved, and a 3-mm full-thickness hole was punched with a sterile disposable biopsy punch (Miltex Instruments, Bethpage, USA). At a given time point, mice were sacrificed and the injured abdominal skin tissues were excised and processed as described below. Serum glucose levels were measured using Accu-Check III glucose analyzer.

### Hematoxylin and eosin (H&E) staining

Mouse abdominal skin tissues were fixed with 4% paraformaldehyde for 1 h, embedded in paraffin and cut into 5 μm serial sections. In brief, corresponding sections were stained with hematoxylin for 5 min. Subsequently, the sections were washed with 1% hydrochloric acid alcohol differentiation liquid for 5 sec and washed with running water for 5 min. Sections were then stained with eosin for 5 min. Images were obtained by Nikon Eclipse TE2000-S microscope (Nikon, Japan) and analyzed by Image Pro Plus 3.1 (Nikon).

### Immunohistochemistry

Immunohistochemistry for Ki67 and involucrin was performed in skin tissue of experimental animals. Antibodies against mouse Ki67 and involucrin were purchased from Abcam and Santa Cruz Biotechnology, respectively. Sections (4 μm) were prepared from formalin fixed paraffin embedded tissue specimens, deparaffinised, and rehydrated in graded alcohols. A heat induced epitope retrieval technique by autoclaving slides for 3 min in 10 mM citric acid buffer was used for detection of Ki67 and involucrin. After quenching endogenous peroxidase with 4% $H_2O_2$ in PBS for 20 min, slides were incubated with primary antibodies at 4℃ overnight (Ki67, 1:100; involucrin, 1:100). Solid phase absorbed

rabbit Ig fraction (DakoCytomation, Denmark) was used to demonstrate specificity of Ki67 and involucrin staining. Visualisation was performed using the LSAB+ kit (DakoCytomation) with 3, 3′-diaminobenzidine as chromogen according to the manufacturer's instructions. Finally, sections were viewed on an Olympus IX70 with Kappa camera and Kappa ImageBase 2.2 software (Kappa opto-electronics GmbH, Germany).

### Caspase 3 activity assay

Caspase3 activity measurement was performed by using a Caspase Assay Kit (Abcam, USA) according to manufacturer's instruction.

### Total RNA extraction and quantitative real-time PCR

Total RNA was isolated from harvested adipose tissues using TRIzol reagent (Invitrogen, USA). RNAs were transcribed to cDNA at 42℃ for 1 h in a 25-μl cocktail containing 5× reverse transcriptase (RT) buffer, 10 mM dNTPs (200 units), Maloney murine leukemia virus reverse transcriptase (MMLV-RT) (Promega, USA), and 100 pmole oligo-dT primer. The concentration of cDNA was estimated by the quantitative RT-PCR method using 2× iQTM SYBR Green Supermix (Bio-Rad, USA) to determine the mRNA level of each gene. Amplification was performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 95℃ for 3 min, followed by 45 cycles at 95℃ for 10 s and 60℃ for 30 s. To confirm PCR specificity, the PCR products were subjected to a melting-curve analysis. The comparative threshold method was used to calculate the relative amounts of mRNA in the experimental samples compared to the control samples. Gene expression was normalized to the expression level of GAPDH. The method of delta-delta cycle threshold (ddCT) was used to calculate the relative fold change of each gene. The following PCR conditions were used: 95℃ for 10 min, followed by 95 for 15 s and 60 for 1 min for 45 cycles. The following oligonucleotide primers were used: human Ki67, forward: 5′-GAGCGGTCCCCACTTTCCCCT-3′ and reverse: 5′-GGAACTG AAATTATGTAATA-3′; human involucrin, forward: 5′-CTCCAC CAAAGCCTCT-3′ and reverse: 5′-CTGCTTAAGCTGCTGC-3′; human loricrin, forward: 5′-GAGAAAAAGCAGCCCACCC-3′ and reverse: 5′-GAACCGCTGCTACCGCCCCC-3′; human filaggrin, forward: 5′-GCCATAATTAATCTTTTCAAG-3′ and reverse: 5′-CAACCATATCTGGGTCATC-3′; human TGFβ1, forward: 5′-CTACTACGCCAAGGAGGTC-3′ and reverse: 5′-TTGCTGAGGTATCGCCAGG-3′; TGFβ2, forward: 5′-GGTCG CGCTCAGCCT-3′ and reverse: 5′-CCTCGATCCTCTTGCGC-3′; human TGFβ3, forward: 5′-AGGATCGAGCTCTTCCAGAT-3′ and reverse: 5′-TGCCA CCGATATAGCGC-3′; human calreticulin, forward: 5′-CGCTTTTATGCTCTGTCGGC-3′ and reverse: 5′-CTCAGGCTTGGAGTCTGTGG-3′; and human β-actin, forward: 5′-GAGCTACGAGCTGCCTGAC-3′ and reverse: 5′-GTAGTTTCGTGGATGCCACA-3′; mouse Ki67, forward: 5′-CGGCTCACCTGGTCACCATC-3′ and reverse: 5′-CCCCGTTTACTTGAGTTGGA-3′; mouse involucrin, forward: 5′-CGGCCAAACCCTGTGAAGGA-3′ and reverse: 5′-GCCCC TGGGGCTCTTGTGGT-3′; mouse TGFβ1, forward: 5′-CTGCT CCCACTCCCGTGGCT-3′ and reverse: 5′- CTTAGTTTGGACA GGATCTG-3′; mouse β-actin, forward: 5′-CTGGTCGTCGACA ACGGCTC-3′ and reverse: 5′-CTCGTCACCCACATAGGAGT-3′. Quantitative real-time PCR experiments were repeated three times independently. Data are represented as the mean ± SEM.

### Western blot analysis

HaCaT cells were harvested, and proteins were extracted with lysis buffer (PRO-PREP; Intron Biotechnology, Korea) for 60 min at 4℃. Protein samples (35 μg) were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Bioscience, USA), and probed with the appropriate primary antibody followed by secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The samples were detected with enhanced chemoluminescence (ECL) kits.

### Transfection using siRNAs for gene silencing

Cells were transfected with 20 nmol/l small interfering (si) RNA oligonucleotides for TGFβ1 and p38 (Santa Cruz Biotechnology) to suppress gene expression. Transfection was performed with Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's directions.

### Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP kit (Abcam) according to manufacturer's direction. Quantitative real time PCR was performed using primers covering AP-1 site in human TGFβ1 promoter (Presser et al., 2013) (forward: 5′-GTCTGCCTCCTGACCCTTCC-3′ and reverse: 5′-CCCCGGCTCCGCCCCGCAAA-3′).

### Enzyme linked immunosorbent assay (ELISA)

Mouse serum CTRP9 levels were measured with each ELISA kit (Aviscera Bioscience, USA) according to the manufacturer's instructions.

### Statistical analysis

All analyses were performed using SPSS/PC statistical program (version 12.0 for Windows; SPSS, USA). Results are presented as the fold of the highest values (mean ± SEM). All of the *in vitro* experiments were performed at least three times. Student's *t* test or two-way ANOVA was used for statistical analysis.

## RESULTS

### CTRP9 suppresses cell growth and differentiation of HaCaT cells

Disordered proliferation and differentiation of keratinocytes result in hyperkeratosis in diabetic ulcers. In clinical examination, abnormal cell proliferation and differentiation are frequently seen in patients with diabetic foot (Kawai et al., 2008). Therefore, we first examined the effects of CTRP9 on proliferation of HaCaT cells. CTRP9 suppressed the uptake of BrdU into HaCaT cells and Ki67 expression in a dose-dependent manner (Figs. 1A and 1B). To determine whether CTRP9 affects the differentiation of keratinocytes, we observed cell morphology and performed quantitative RT-PCR

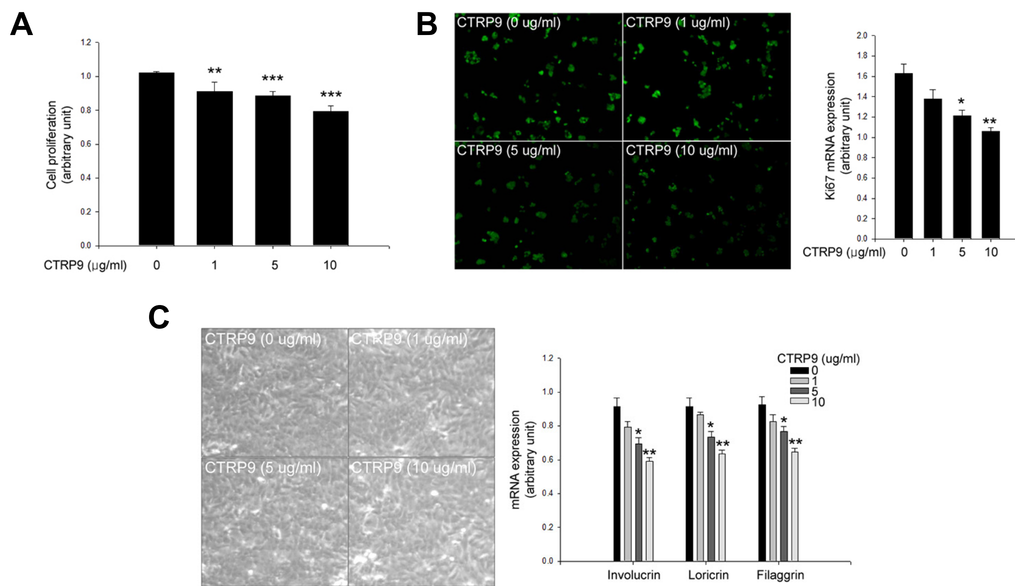



Fig. 1. CTRP9 suppresses proliferation and differentiation of human keratinocytes. (A) Proliferation assay in HaCaT cells cultured in the presence of CTRP9 (0-10 μg/ml) for 24 h. Cell proliferation was assessed by determining BrdU uptake. (B) Ki67 expression was detected by immunofluorescence. Quantitative real-time PCR analysis of *Ki67* mRNA expression in HaCaT cells treated with CTRP9 for 18 h. (C) Inverted microscopy images of HaCaT cells treated with CTRP9 for 18 h. Quantitative real-time PCR analysis of *involucrin*, *loricrin*, *filaggrin* mRNA expression in HaCaT cells treated with CTRP9 for 18 h. β-Actin was used as an internal standard. Mean ± SEM were calculated from three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with levels in control.

to measure the mRNA expression of involucrin, loricrin, and filaggrin, well-known markers of keratinocyte differentiation. CTRP9 suppressed differentiation markers' mRNA expression in a dose-dependent manner, although it did not affect cell morphological change (Fig. 1C). To confirm a more certain cell morphological change in the suppressive effect of CTRP9 on differentiation, we treated HaCaT cells with calcium chloride ($CaCl_2$) which is well-known to stimulate differentiation of HaCaT cells (Deyrieux and Wilson, 2007). Within 24 h after 2.8 mM $CaCl_2$ exposure, HaCaT cells exhibited a more cuboidal shape with cell-cell tight junction formation. However, CTRP9 treatment reversed these changes. Furthermore, CTRP9 decreased $CaCl_2$-induced differentiation markers' mRNA expression in a dose-dependent manner (Supplementary Fig. S1).

## CTRP9 causes apoptosis in HaCaT cells

The epidermis undergoes a continuous turnover through whole life, in which keratinocytes migrate to the upper layer, leading to final cell differentiation and keratinization. This process is known as apoptosis (Kawai et al., 2008). Therefore, apoptosis in keratinocytes plays a crucial role in the regulation of epidermal development through morphological and biochemical changes. Thus, we next examined the effect of CTRP9 on apoptosis of HaCaT cells by performing MTT viability assays and caspase 3 activity assays. Our results demonstrated that CTRP9 induced apoptosis of HaCaT cells in a dose-dependent manner (Figs. 2A and 2B). Calreticulin has been reported to regulate the clearance of early apoptotic bodies through adiponectin-mediated regulation (Takemura et al., 2007) and its expression is augmented by adiponectin treatment in HaCaT cells (Kawai et al., 2008) implying that it plays an important role in the wound healing. Therefore, we investigated the effect of CTRP9 on calreticulin mRNA expression. CTRP9 augmented calreticulin mRNA expression in a dose-dependent manner (Fig. 2C).

## CTRP9 specifically induces TGFβ1 mRNA expression and consequently promotes p38 phosphorylation in HaCaT cells

As TGFβs have been documented to play an important role in wound healing in all cell types (Frank et al., 1996; O'Kane and Ferguson, 1997), we investigated the effects of CTRP9 on *TGFβ* mRNA expression in HaCaT cells. As shown in Fig. 3A, CTRP9 increased *TGFβ1* mRNA expression in a dose-dependent manner; however, *TGFβ2* and *TGFβ3* mRNA expression was not changed by CTRP9 treatment. Since transcription factor AP-1 regulates TGFβ1 (Kim et al., 1989), we performed ChIP assay to assess whether AP-1-mediated TGFβ1 promoter binding activity can be modulated by CTRP9. CTRP9 induced AP-1 binding activity in a dose-dependent manner (Fig. 3B). Mitogen-activated protein kinases (MAPKs), including JNK, p38, and ERK1/2, are known to be involved in stress-induced cell proliferation, migration, and apoptosis (Kyriakis and Avruch, 2001; Xia et al., 1995; Zhan et al., 2003) and to be regulated by TGFβ1 (Jin et al., 2016; Liu et al., 2012; Takekawa et al., 2002). Therefore, we next examined the effects of CTRP9 on MAPK phosphorylation. Treatment of HaCaT cells with CTRP9 significantly and specifically induced p38 phosphorylation.

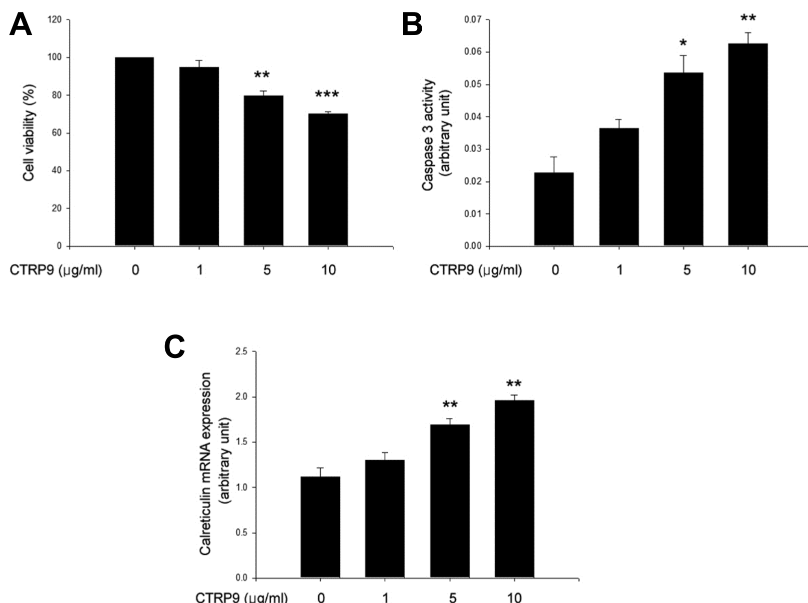



Fig. 2. CTRP9 induces apoptosis in human keratinocytes. (A) Cell viability in HaCaT cells treated with CTRP9 (0-10 μg/ml) for 24 h was assessed by MTT assay. (B) Measurement of caspase 3 activity in HaCaT cells treated with CTRP9 (0-10 μg/ml) for 24 h. (C) Quantitative real-time PCR analysis of *calreticulin* mRNA expression in HaCaT cells treated with CTRP9 for 18 h. The β-Actin was used as an internal standard. Mean ± SEM were calculated from three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with the control.

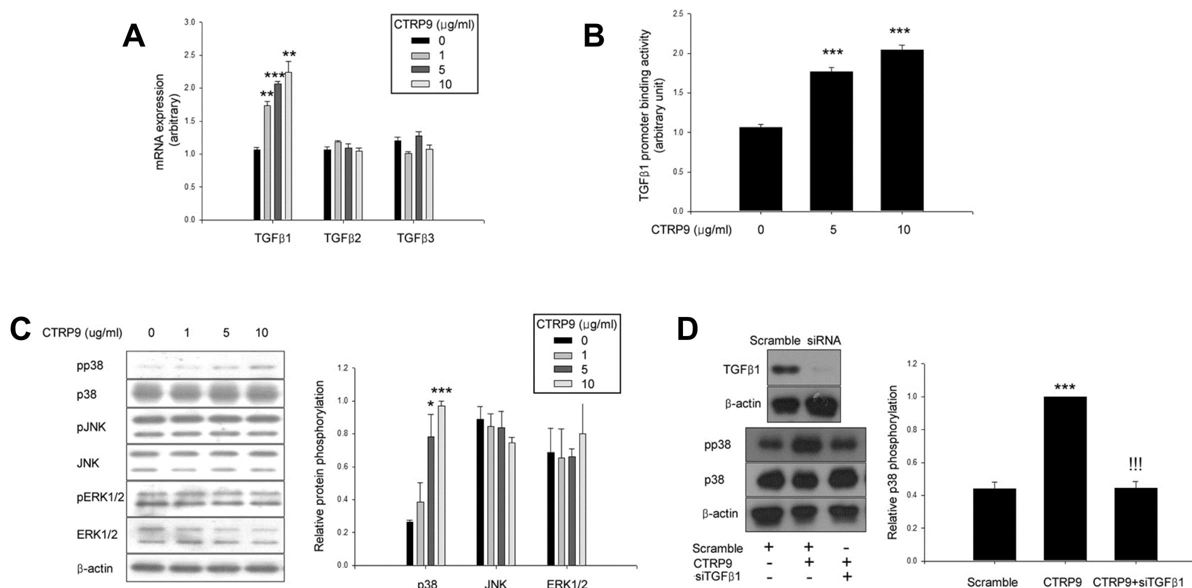



Fig. 3. CTRP9 increases *TGFβ1* mRNA expression and p38 phosphorylation in keratinocytes. (A) Quantitative real-time PCR analysis of TGFβ1, TGFβ2, and TGFβ3 mRNA expression in HaCaT cells treated with CTRP9 (0-10 μg/ml) for 18 h. (B) AP-1 binding to the TGFβ1 promoter was determined by ChIP assay in HaCaT cells treated with CTRP9 (0-10 μg/ml) for 18 h. (C) Western blot analysis of JNK, p38, and ERK1/2 phosphorylation in HaCaT cells treated with CTRP9 (10 μg/ml) for 18 h. (D) Scramble siRNA or TGFβ1 siRNA-transfected HaCaT cells were treated with 10 μg/ml CTRP9 for 18 h. Cell extracts were applied to Western blot analysis to measure p38 phosphorylation. Total forms and β-actin were used as internal standards. Mean ± SEM were calculated from three independent experiments. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared with the control. $^{!!!}P < 0.001$ compared with CTRP9 treatment.

Furthermore, suppression of TGFβ1 expression markedly abrogated the effects of CTRP9 on p38 phosphorylation (Figs. 3C and 3D), suggesting that CTRP9 promotes p38 phosphorylation through a TGFβ1-mediated pathway in keratinocytes.

### CTRP9 prevents proliferation and differentiation and induces apoptosis of HaCaT cells via TGFβ1-mediated induction of p38 phosphorylation

The p38-mediated cascade plays a key role in suppressing cell proliferation (Saika et al., 2004) and differentiation

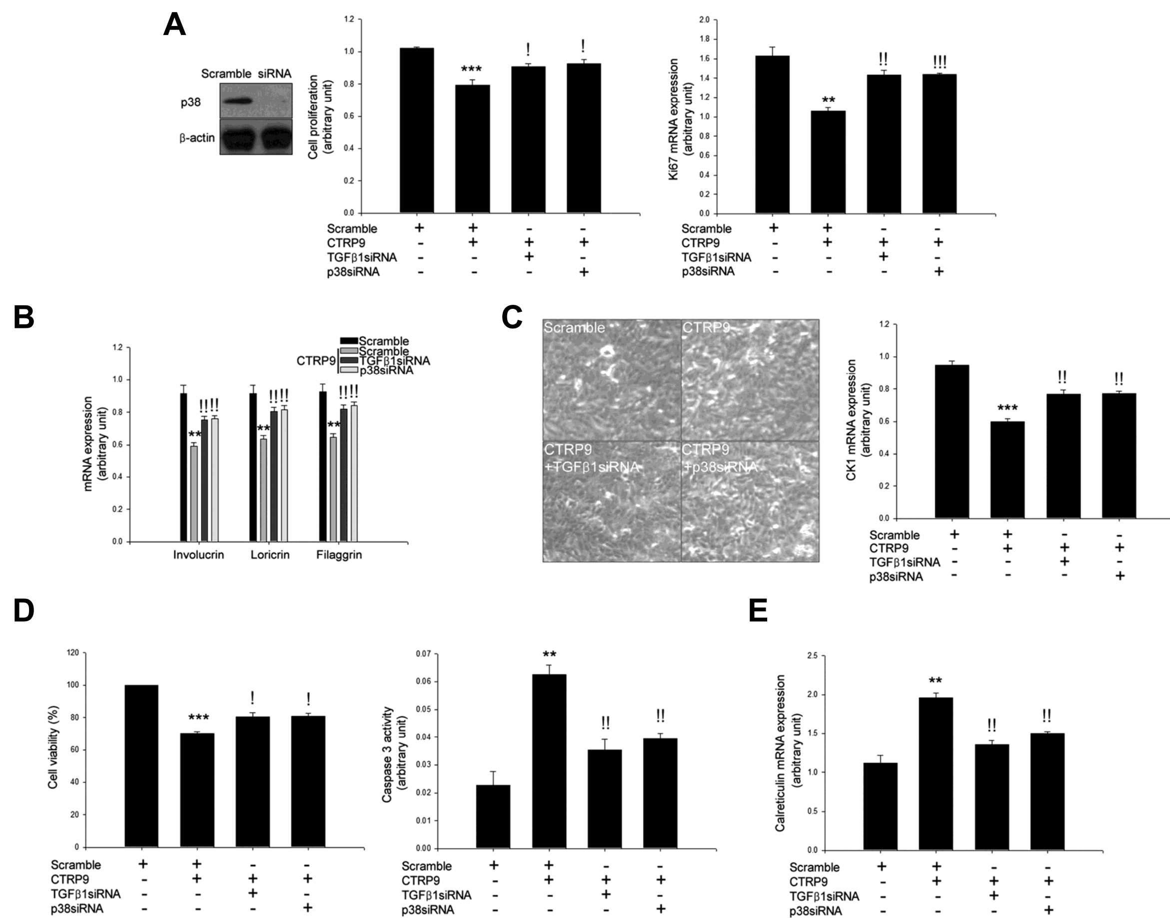
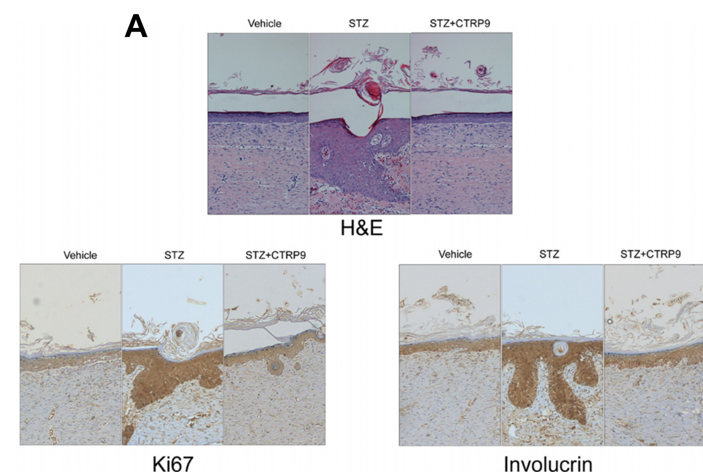



Fig. 4. CTRP9 regulates proliferation, differentiation, and apoptosis in keratinocytes through TGFβ1-mediated induction of p38 phosphorylation. (A) Proliferation assay. quantitative real-time PCR analysis of *Ki67* mRNA expression, (B) quantitative real-time PCR analysis of *involucrin*, *loricrin*, *filaggrin* mRNA expression, (C) inverted microscopy images. Quantitative real-time PCR analysis of *CK1* mRNA expression, (D) cell viability assay, (E) measurement of caspase 3 activity, and (E) quantitative real-time PCR analysis of *calrecticulin* mRNA expression in HaCaT cells transfected with siRNA specific for TGFβ1 or p38 and treated with CTRP9 for 18 or 24 h. β-actin was used as internal standards. Mean ± SEM were calculated from three independent experiments. ***$P < 0.001$ and **$P < 0.01$ compared with the control. !!$P < 0.01$ and !$P < 0.05$ compared with CTRP9 treatment.

through inhibition of involucrin promoter activity (Efimova et al., 1998). Furthermore, activation of p38 is required for induction of apoptosis (Kang et al., 2003). We therefore examined the effects of siRNA specific for TGFβ1 and p38 on the activity of CTRP9 in HaCaT cells. As shown in Fig. 4, suppression of TGFβ1 or p38 expression markedly abrogated the effects of CTRP9 on proliferation, differentiation, hyperkeratosis, and apoptosis of HaCaT cells (Figs. 4A-4D). Knockdown of TGFβ1 or p38 also significantly suppressed CTRP9-induced calreticulin mRNA expression (Fig. 4E). To verify a more certain cell morphological change in the suppressive effect of CTRP9 on hyperkeratosis, we treated HaCaT cells with arsenic which is reported to stimulate CK1 expression in HaCaT cells (Sun et al., 2009). CTRP9 treatment ameliorated arsenic-induced cell cluster formation. However, knockdown of TGFβ1 or p38 abrogated the suppressive effects of CTRP9 (Supplementary Fig. S2).

### CTRP9 injection suppressed STZ-induced epidermal thickening in abdominal skin tissue of mice after wound healing

Mice with non-fasting blood glucose above 250 mg/dl were considered as diabetic mice (Pavlinkova et al., 2008). In this study, intravenous injection of CTRP9 did not attenuate STZ treatment-induced hyperglycemia in mice (Supplementary Fig. S3A). Furthermore, STZ treatment decreased serum CTRP9 levels in mice. However, CTRP9 injection restored significantly these changes (Supplementary Fig. S3B). As shown in Fig. 5, epidermal thickening was observed in STZ-treated mice after wound healing. Furthermore, STZ treatment augments both Ki67 and involucrin expression (brown area). However, CTRP9 injection via tail vein markedly restored these changes (Figs. 5A and 5B). CTRP9 administration markedly increased TGFβ1 mRNA and caspase 3 activity (Fig. 5C).



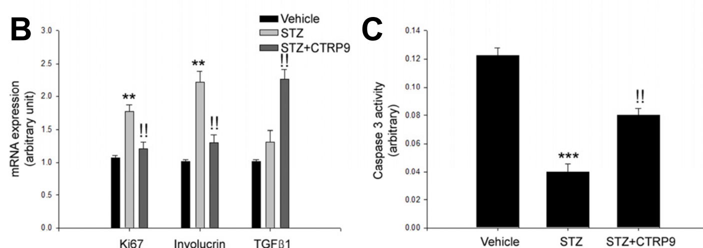


Fig. 5. Immunohistochemical staining for Ki67 and involucrin in abdominal skin tissue of experimental mice. (A) Representative histological analysis of abdominal skin tissue of experimental mice. Sections were stained with hematoxylin and eosin (H&E) (upper). Immunohistochemical staining for Ki67 (left panel) and involucrin (right panel) in sections of the abdominal skin tissue from experimental mice. Ki67 and involucrin were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB, *brown*). (B, C) Quantitative real-time PCR analysis of *Ki67*, *involucrin*, and *TGFβ1* mRNA expression (B) and measurement of caspase 3 activity (C) in abdominal skin tissue of experimental mice. The β-actin was used as internal standards. Mean ± SEM were calculated from five experimental mice. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared with the control. $^{!!}P < 0.01$ compared with STZ treatment.

Fig. 6. Schematic diagram of the possible mechanism for the effects of CTRP9 on proliferation, differentiation, and apoptosis in human keratinocytes.

## DISCUSSION

The epidermis of the skin forms the outermost layer of the body, and one of its most important roles is protection from the external environment. Consequently, the skin tends to be easily injured by external factors. The process of wound healing is tightly controlled through biochemical signal transduction. This repair system is significantly impaired in patients with diabetes, leading to severe diabetic ulcers; however, the mechanism underlying the impaired healing in diabetes remains to be elucidated.

CTRP9 is a novel adipokine that is exclusively expressed in adipocytes and shares closest amino acid identity (54%) with adiponectin in the globular domain (Seldin et al., 2014). Serum CTRP9 level is decreased in patients with hyperglycemia and insulin resistance (Hwang et al., 2014). Adenovirus-mediated overexpression of CTRP9 attenuated non-alcoholic fatty liver disease through AMPK-autophagy-mediated suppression of ER stress in mice (Jung et al., 2015). Furthermore, CTRP9 has been suggested to be a regulator of vascular function in rodents (Uemura et al., 2013; Zheng et al., 2011). In clinical trials, serum CTRP9 concentration was significantly correlated with arterial stiffness in patients with type 2 diabetes (Jung et al., 2014).

The protective effects of CTRP9 on metabolic disorders have been well documented through several reports (Jung et al., 2015; Peterson et al., 2013; Wei et al., 2014; Zheng et al., 2011). However, the effects of CTRP9 on wound healing of the epidermis under diabetic conditions are not well understood, although Kawai et al. (2008) have reported beneficial effects of adiponectin on impaired wound healing in diabetes. Previous studies have shown that adiponectin inhibits the proliferation and migration of human smooth muscle cells (Arita et al., 2002; Matsuda et al., 2002). Wang et al. (2005) have demonstrated that adiponectin suppresses the proliferation of human smooth muscle cells via interaction with several growth factors, such as heparin binding-epidermal growth factor-BB (HB-EGF), platelet-derived growth factor-BB (PDGF-BB), and basic fibroblast growth factor (bFGF). Especially, CTRP9 attenuates neointimal formation caused by vascular injury through inhibition of vascular smooth muscle cell growth via a cAMP-dependent pathway (Uemura et al., 2013).

In this study, we demonstrated for the first time that CTRP9 suppresses the proliferation and differentiation of keratinocytes through induction of TGFβ1 expression. These results might be controversial because re-epithelialization generally results from proliferation, migration, and differentiation of keratinocytes in the wound healing process. However, hyperkeratinization can occur in areas surrounding ulcers in patients with diabetic foot, suggesting that this process might not be regulated by signaling pathways associated with proliferation and differentiation. Although the mechanisms of hyperkeratinization or callosity-mediated mechanisms in diabetic foot remain unclear, a decrease in serum CTRP9 in patients with diabetes might be closely associated with impairment of wound healing.

TGFβs are important regulators of cell proliferation, differentiation, and other functions in many cell types (Bierie and Moses, 2006; Massague et al., 2000). Mammals possess three isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3 (Khalil, 1999). Among these, TGFβ1 expression is rapidly augmented in keratinocytes and macrophages after skin injury (Singer and Clark, 1999). In this study, we found that CTRP9 induces TGFβ1 expression in HaCaT cells, whereas expression of TGFβ2 and TGFβ3 was not affected by CTRP9 treatment. Since TGFβ1 has been considered a negative regulator of cell growth and migration (Koch et al., 2000; O'Kane and Ferguson, 1997), it might contribute to the suppressive effects of CTRP9 on the proliferation of keratinocytes. In addition, we investigated the regulation mechanism in CTRP9-mediated TGFβ1 induction. CTRP9 has been reported to stimulate AMPK phosphorylation (Jung et al., 2015; Kambara et al., 2012; Wong et al., 2009). It has been reported that transcription factor AP-1 is involved in ionizing radiation-mediated induction of TGFβ1 expression (Gault et al., 2002). Furthermore, AMPK activation by AICAR enhances AP-1-mediated gene regulation (Iwasaki et al., 2007). In this study, we found that treatment of HaCaT cells with CTRP9 increased TGFβ1 mRNA expression in a dose-dependent manner and AP-1 binding activity in TGFβ1 promoter. Moreover, we also found that CTRP9 treatment induced AMPK phosphorylation in HaCaT cells (Supplementary Fig. S4). Therefore, we expected that CTRP9 may augment TGFβ1 mRNA expression through AMPK/AP-1-dependent pathway. Further studies are required to investigate the role of AMPK in CTRP9-induced TGFβ1 expression.

In various cell types, MAPKs such as p38, JNK, and ERK1/2 are known to be involved in cell proliferation, migration, and survival (Nebreda and Porras, 2000; Wei et al., 2010; Zhan et al., 2003). Among these MAPKs, p38 was markedly phosphorylated after CTRP9 treatment in HaCaT cells. On the basis of previous reports (Ferrari et al., 2012; Wu et al., 2009), we further evaluated whether CTRP9-induced TGFβ1 affects the phosphorylation of p38. Suppression of TGFβ1 significantly abrogated the effects of CTRP9 on the phosphorylation of p38, as well as cell proliferation and differentiation, and also induced apoptosis. These results suggest that CTRP9 suppresses proliferation and differentiation and induces apoptosis through TGFβ1-associated induction of phosphorylation of p38 in keratinocytes.

Hyperkeratosis is a phenomenon in which the stratum corneum becomes thickened as a result of abnormal accumulation of keratin. Hyperkeratosis in individuals with diabetes often occurs on specific areas such as the soles or malleoli and is thought to lead to abnormal proliferation, differentiation, and apoptosis of keratinocytes. In the current study, we showed that CTRP9 induced apoptosis in HaCaT cells. However, these results seem to be controversial because CTRP9 also inhibited the differentiation of keratinocytes, as shown in Fig. 1C. The reason for this is unclear, although the effects of CTRP9 might be dependent on the differentiation stage of keratinocytes. As calrecticulin has been reported to regulate the removal of apoptotic bodies through binding to adiponectin (Takemura et al., 2007), we further examined the effects of CTRP9 on calrecticulin expression in HaCaT cells and found that CTRP9 induced calrecticulin mRNA expression in a dose-dependent manner. These results suggest that CTRP9 has anti-hypertrophic effects on the epidermis. To validate results of *in vitro* experiments, we also performed *in vivo* experiments using a type 1 diabetic animal model. Instead of applying CTRP9 to the wound site, mouse recombinant CTRP9 was injected via tail vein, because we hypothesized that hyperglycemia-mediated decrease of circulating concentrations of CTRP9 produced by adipocytes might cause impairment of wound healing. In the current study, STZ-induced hyperglycemia thickened epidermis layers in injury site of mice. Similar to high fat diet effects on serum CTRP9 in mice (Wang et al., 2016), we found that STZ treatment reduced circulating CTRP9 levels (Supplementary Fig. S3B). CTRP9 supplementation to STZ-treated mice suppressed epidermal thickening, Ki67 and involucrin expression in abdominal skin tissue. Conversely, it augmented TGFβ1 mRNA expression and caspase 3 activity as *in vitro* results. These results suggest that circulating CTRP9 may attenuate hyperglycemia-induced epidermal thickening through suppression of keratinocyte proliferation. In this study, unfortunately, significant hyperkeratosis was not observed in abdominal skin tissue of STZ-treated mice. Therefore, in order to better investigate the effect of CTRP9 on diabetic wound, future *in vivo* experimental conditions for development of hyperkeratinization by STZ treatment should be established. Thereafter, we will examine the effects of CTRP9 via tail vein injection on wound healing or apply recombinant CTRP9 to the injury. Additionally, further studies are also required to identify novel CTRP9 inducers. Therefore, we are now preparing to investigate CTRP9 promoter studies and screen many kinds of drugs and natural compounds for inducing CTRP9 production in adipocytes as well as develop an ointment containing CTRP9 as an external medicine.

In conclusion, we show that CTRP9 suppresses proliferation and differentiation and causes apoptosis of keratinocytes through TGFβ1-mediated induction of a p38-dependent pathway.

## CONCLUSIONS

CTRP9 upregulates the expression of TGFβ1 via AP-1-mediated transcriptional regulation. This upregulation resulted in reduced cell proliferation, differentiation, and apoptosis through p38-mediated pathway in human

keratinocytes (Fig. 6).

CTRP9-mediated activation of the TGFβ1-p38-dependent pathway is a potential therapeutic target for the treatment of diabetic foot ulcer. Future studies employing TGFβ1 and p38 knockout in diabetic foot ulcer animal models may help elucidate the specific roles of TGFβ1 and p38 in CTRP9-mediated wound healing.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

## ACKNOWLEDGMENTS

The National Research Foundation of Korea (NRF) grant funded by the Korea government Ministry of Science, ICT & Future Planning Contract grant number: 2016R1C1B2012674.

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